Both orexin receptors are expressed in rat ovaries and fluctuate with the estrous cycle: effects of orexin receptor antagonists on gonadotropins and ovulation

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Silveyra P, Lux-Lantos V, Libertun C. Both orexin receptors are expressed in rat ovaries and fluctuate with the estrous cycle: effects of orexin receptor antagonists on gonadotropins and ovulation. *Am J Physiol Endocrinol Metab* 293: E977–E985, 2007. First published July 17, 2007; doi:10.1152/ajpendo.00179.2007.—Orexins are peptides controlling feeding, sleep, and neuroendocrine functions. They are synthesized by the hypothalamus with projections throughout the brain. Orexins and their orexin 1 (OX_1) and orexin 2 receptors (OX_2) are present outside the central nervous system. Here the expression of preproorexin (PPO), OX_1, and OX_2 was studied in rat ovaries. PPO, OX_1, and OX_2 were determined by quantitative real-time RT-PCR in ovaries of cycling Sprague-Dawley rats on all days of the cycle. Serum hormones and food consumption were determined. Ovarian OX_1 and OX_2 expression was then studied after ovulation blockade with Cetrorelix or Nembutal. Finally, proestrous rats were treated at 1400 and 1900 with a selective OX_1 antagonist (SB-334867-A) and/or a selective OX_2 antagonist (JNJ-10397049), and hormone levels, ovulation, and ovarian histology were studied. Both receptors' expression increased in the ovary between 1700 and 2300 of proestrus exclusively, in coincidence with hormone peaks, but not with the dark-light cycle or food intake. PPO was not detected. Cetrorelix or Nembutal prevented the increases of OX_1 and OX_2 while blunting gonadotropin peaks. SB-334867-A and JNJ-10397049, alone or combined, decreased serum gonadotropins and reduced ova number the following morning; ovaries showed a bloody (hyperemic and/or hemorrhagic) reaction with more preovulatory follicles and less corpora lutea. Here we demonstrate for the first time an increased ovarian expression of both OX_1 and OX_2, only during proestrous afternoon, and its hormone dependence but not dependence on the dark-light cycle. Two new receptor antagonists reduced proestrous gonadotropins and/or ova number while producing ovarian structural changes.

orexin 1 and orexin 2 receptor antagonists; ovary

OREXIN-A AND OREXIN-B (hypocretins A and B), via their orexin-1 (OX_1) and orexin-2 (OX_2) receptors, are regulatory peptides controlling feeding, sleep-wakefulness, and a variety of neuroendocrine and autonomic functions. Both neuropeptides are formed by cleavage from a 130-amino acid precursor, preprorexin (PPO), which was isolated from rat hypothalamus (14, 15, 17, 21, 34, 38, 50). Both neuropeptides are synthesized by neurons in the lateral hypothalamus with projections throughout the brain (20, 40, 45, 62). Orexin A, a 33-amino acid peptide, and orexin B, a linear peptide of 28 amino acids, are potent agonists at both the OX_1 and OX_2 G protein-coupled receptors. The structure of orexins and their receptors is highly conserved (35).

The widespread distribution of mRNAs encoding OX_1 and OX_2 in the brain suggests that orexins are involved in multiple functional pathways, including their participation in the brain control of the pituitary. Brain orexins have been implicated in the control of gonadotropin-releasing hormone (GnRH) neurons and in the secretion of pituitary gonadotropins, in a variety of in vivo and in vitro experiments, in rodents (8, 18, 23, 32, 47, 49, 51, 53, 54, 57), sheep (22), and humans (33). Also, modifications of the brain orexinergic system have been associated with the estrous cycle, pregnancy, and lactation (5, 7, 19, 27, 46, 53, 60).

Orexins and their receptors have been described outside the central nervous system (CNS). Receptors are expressed in glands such as the pituitary (4, 13, 25, 26, 53), adrenal (25, 29), thyroid (26), and pancreatic islets (41). In human peripheral tissues, immunoreactivity for orexin A was detected in ganglion cells, myenteric plexuses, gastrointestinal endocrine cells, islet cells of the pancreas, placental syncytiotrophoblasts, and decidual cells; mRNA expression for PPO was detected in the kidney, adrenal gland, pancreas, placenta, stomach, and intestinal epithelial cells (39). Immunoreactive orexin A in human plasma was found by radioimmunoassay (RIA) (2). Regarding the gonads, orexin A and B immunoreactivity has been detected in rat testis (3); OX_1 and OX_2 expression was reported in testis, epididymis, penis, and seminal vesicle, whereas PPO was reported only in the epididymis and penis in the human male reproductive system (28). In a screening study comparing several tissues of male and female rats, a very low amount of OX_1 mRNA was found in pooled ovaries (26).

Taking in consideration these data and our recent results (53), we decided to study PPO, OX_1, and OX_2 expression in ovaries of adult rats at different stages of the estrous cycle and correlated them to hormonal status, dark-light cycle, and food consumption. Furthermore, the effects of two potent and selective antagonists of orexin receptors OX_1 and OX_2 were evaluated on gonadotropin secretion, ovarian histology and ovulation.

MATERIALS AND METHODS

Animals. Adult female virgin Sprague-Dawley rats (200–250 g) from the Instituto de Biología y Medicina Experimental colony were

housed in groups in an air-conditioned room (21°C), with lights on from 0700 to 1900. They were given free access to laboratory chow and tap water. All studies on animals were performed according to protocols for animal use approved by the Institutional Animal Care and Use Committee (Instituto de Biología y Medicina Experimental, Consejo Nacional de Investigaciones Científicas y Técnicas) and by the National Institutes of Health.

First set of experiments. Regular cycling female rats were killed by decapitation at 1100, 1500, 1700, 1900, and 2300 in all stages of the estrous cycle. The stage of the cycle was determined by vaginal smears for two consecutive weeks. Regular cycles were defined as the occurrence of three consecutive 4-day cycles. Occurrence of ovulation was evaluated by microscopic examination of the oviduct the morning of estrus.

In all cases trunk blood was collected, and sera were stored at −20°C for hormone determinations by RIA. Day and night food intake was also recorded. For this purpose, during the entire experiments rats were kept in individual cages, and food consumption was determined at 0700 and 1900.

Ovaries were rapidly removed and placed on ice for dissection; tissue samples were immediately homogenized in TRIzol reagent (Invitrogen) and kept at −70°C until used. Levels of expression of mRNAs for PPO, OX_1, and OX_2 in the ovaries were determined by quantitative real-time RT-PCR.

Second set of experiments. In a group of proestrous rats, ovulation was blocked, as described (53). Briefly, Cetrorelix, a GnRH receptor antagonist (Serono, Buenos Aires, Argentina), 100 μg/100 μl sterile water ip per rat, or pentobarbital sodium (Nembutal), a barbiturate that blocks ovulation, 30 mg/kg body wt ip, diluted in 1:7:2 parts of absolute ethanol, sterile water, and propylene glycol, respectively, or saline as control, was injected at 1400 of proestrus. Animals were decapitated at 1900, and ovaries and sera collected. Ovarian levels of expression of OX_1 and OX_2 were determined by real-time RT-PCR.

Third set of experiments. Finally, groups of cycling rats were treated at 1400 and 1900 of proestrus with the OX_1 and/or OX_2 blocking drugs. SB-334867-A, a nonpeptide OX_1-selective receptor antagonist (Tocris Bioscience) (55), was injected intraperitoneally at the dose of 10 mg/kg body wt diluted in 50% DMSO and 50% saline. JNJ-10397049, a selective OX_2 antagonist consisting of a substituted 4-phenyl-[1,3]dioxane [(9,1-(2,4-dibromo-phenyl)-3-((4S,5S)-2,2dimethyl-4-phenyl-[1,3]dioxan-5-yl)-urea) (37) provided by Johnson and Johnson Pharmaceutical Research and Development, San Diego, CA], was injected intraperitoneally at the dose of 10 mg/kg body wt diluted in 50% DMSO and 50% saline. A third group was injected simultaneously with both blocking drugs: 10 mg/kg of SB-334867-A plus 10 mg/kg of JNJ-10397049. Control ovulating proestrous rats received two injections of vehicle. In addition, two other groups of proestrous rats were injected with Cetrorelix or Nembutal (as in the 2nd set of experiments) at 1400 and with saline at 1900, as controls of nonovulating animals. Blood samples were collected at 1900 from the jugular vein under light ether anesthesia, before the second injection of the blocking drug or saline, according to the respective protocol. Rats were decapitated at 0900 the following morning, and ovaries and sera were collected and kept as above.

Food intake was determined in all groups at 1400 and 1900 of proestrus to evaluate the effectiveness of OX_1 and OX_2 antagonists.

Ovarian morphology. To evaluate changes in the macro- and microstructure, some ovaries of each group were first examined after laparotomy and then immediately fixed in 5% neutral buffered formaldehyde for histological examination. After embedded in paraffin, ovary sections were stained with hematoxylin-eosin to count the number of preovulatory follicles and corpora lutea under a light microscope as described by Parborell et al. (43) and Peluffo et al. (44).

Total RNA preparation and cDNA synthesis. Total RNA was isolated using the TRIzol reagent method from tissue homogenates. The RNA concentration was determined based on absorbance at 260

nm, and its purity was evaluated by the ratio of absorbance at 260 nm/280 nm (>1.8). RNAs were kept frozen at −70°C until analyzed.

After digestion of genomic DNA by treatment with deoxyribonuclease I (Ambion, Austin, TX), first-strand cDNA was synthesized from 1 μg of total RNA in the presence of 10 mM $MgCl_2$, 50 mM Tris·HCl (pH 8.6), 75 mM KCl, 0.5 mM deoxy-NTPs, 1 mM DTT, 1 U/μl RnaseOUT (Invitrogen), 0.5 μg oligo(dT)$_{15}$ primer (Biodynamics, Buenos Aires, Argentina), and 20 U MMLV reverse transcriptase (Epicentre, Madison, WI). To validate successful deoxyribonuclease I treatment, the reverse transcriptase was omitted in control reactions. The absence of PCR-amplified DNA fragments in these samples indicated the isolation of RNA free of genomic DNA.

Quantitative real-time PCR. Sense and antisense oligonucleotide primers were designed based on the published cDNA PPO, OX_1 and OX_2 receptor, and cyclophilin sequences using the PrimerExpress software (Applied Biosystems, Foster City, CA). Oligonucleotides were obtained from Invitrogen. The sequences of the primer were as follows: PPO sense, GCCTCAGACTCCTTGGGTATTTG; PPO antisense, GGCAATCCGGAGAGATGGT; OX_1 sense, GCCTGCCAGCCTGTTAGTG; OX_1 antisense, CAAGGCATGGCCGAAGAG; OX_2 sense, GAAAGAATATGAGTGGGTCCTGATC; OX_2 antisense, CAGGACGTTCCCGATGAGA; cyclophilin sense, GTGGCAAGATCGAAGTGG; and cyclophilin antisense, TAAAAATCAGGCCTGTGG.

Quantitative measurements of PPO, OX_1 and OX_2 receptor, and cyclophilin cDNA were performed by kinetic PCR using SYBR green I as fluorescent dye (Invitrogen). PCR reaction consisted of 100 ng cDNA, 0.4 μM primers, 10 mM Tris·HCl, 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM deoxy-NTPs, and 1.25 U *Taq* polymerase (Invitrogen) in a final volume of 25 μl. After denaturing at 95°C for 5 min, the cDNA products were amplified with 40 cycles, each cycle consisting of denaturing at 95°C for 15 s, annealing at 62°C for 30 s, and extension at 72°C for 30 s. The accumulating DNA products were monitored by the ABI7500 sequence detection system (Applied Biosystems), and data were stored continuously during the reaction. The results were validated based on the quality of dissociation curves, generated at the end of the PCR runs by ramping the temperature of the samples from 60°C to 95°C, meanwhile continuously collecting fluorescence data. Product purity was confirmed by polyacrylamide gel electrophoresis. Each sample was analyzed in duplicate along with specific standards and no-template controls to monitor contaminating DNA.

The calculations of the initial mRNA copy numbers in each sample were made according to the cycle threshold (C_t) method. The C_t for each sample was calculated at a fluorescence threshold (R_n) using the ABI7500 sequence detection system software with an automatic baseline setting. For all designed primer sets, linearity of real-time
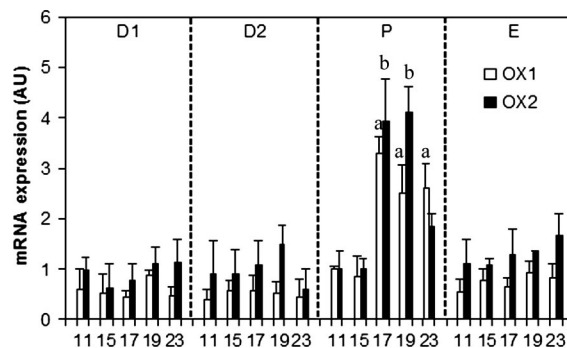

Fig. 1. Orexin 1 (OX_1) and orexin 2 receptor (OX_2) mRNA expression in rat ovary at different times of diestrus [diestrus 1 (D1) and diestrus 2 (D2)], proestrus (P), and estrus (E). AU, arbitrary units. [a,b] Different for all other times and all other days ($P < 0.05$) ($n = 4$–8). Lights on at 0700; lights off at 1900.

Table 1. *Food intake during estrous cycle*

Cycle Stage	%Food Intake/Body Weight, g/g					
	0700–1100	1100–1500	1500–1700	1700–1900	1900–2300	2300–0700
D1	0.22±0.14	0.28±0.11	0.10±0.07	0.56±0.16	1.87±0.12	3.55±0.32
D2	0.21±0.15	0.20±0.10	0.32±0.11	0.46±0.20	1.74±0.23	3.90±0.28
P	0.34±0.14	0.27±0.16	0.18±0.08	0.50±0.18	1.62±0.12	3.99±0.30
E	0.09±0.09	0.20±0.11	0.21±0.10	0.48±0.15	1.39±0.08	3.50±0.32

Values are means $\pm$ SE; $n = 7$–9. Results are expressed in % of food intake (g)/ body weight (g). Diurnal (0700 to 1900) and nocturnal (1900 to 0700) food consumption of female rats is shown in all stages of the estrous cycle at 2- and 4-h intervals. D1 and D2, diestrus 1 and 2, respectively; P, proestrus; E, estrus. Diurnal food intake was significantly lower than nocturnal ($P < 0.001$). Nocturnal food consumption was significantly lower in E than D2 ($P < 0.05$).

RT-PCR signaling was determined with wide-range serial dilutions of reference cDNA that covered the amount of target mRNA expected in the experimental samples, and clear linear correlations were found between the amount of cDNA and the C_t for the duration of at least 40 real-time RT-PCR rounds.

For each target gene, the relative gene expression was normalized to that of cyclophilin housekeeping gene by use of the standard curve method, as described by the manufacturer (User Bulletin 2). Results are expressed as arbitrary units (AU) for comparison among samples. AU is defined as the expression level relative to a sample of 1100 of proestrus or saline (calibrator sample).

Membrane preparation and Western blot analysis. Anterior and mediobasal hypothalamus, frontoparietal cortex, and ovaries from rats were rapidly removed and frozen. The membrane fraction was isolated according to the method of Olpe et al. (42). Briefly, tissues were homogenized in 10 volumes of ice-cold 0.32 M sucrose, containing 1 mM $MgCl_2$ and 1 mM K_2HPO_4. Homogenates were centrifuged at 750 g, the pellet was resuspended, and the centrifugation was repeated. The supernatants were pooled and centrifuged at 18,000 g for 15 min. The pellet was osmotically shocked, centrifuged at 39,000 g, resuspended in 50 mM Tris·HCl, 2.5 mM $CaCl_2$, pH 7.4 (10 vol/g of original tissue), and washed twice. Membranes were frozen at $-70°C$.

SDS-10% polyacrylamide gel electrophoresis was then carried out on 50 mg of each of the membrane preparations. Proteins were transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech). Blots were incubated with the polyclonal rabbit antibodies (OX1R11-A, Alpha Diagnostics, 1:1,000 or OX2R21-A, Alpha Diagnostics, 1:800) overnight at 4°C followed with a peroxidase-conjugated goat anti-rabbit secondary antibody (Santa Cruz, 1:4,000, 1 h at room temperature).

Immunoreactive bands were detected using the Western blotting chemiluminescence Luminol Reagent (Santa Cruz Biotechnology).

Hormonal determinations. Serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), and prolactin (PRL) were estimated by RIA using kits provided by the National Institute of Diabetes and Digestive and Kidney Diseases. Results were expressed in terms of RP_3 rat LH, FSH, and PRL standards. Assay sensitivities were 0.015 ng/ml for LH, 0.1175 ng/ml for FSH, and 1.6 ng/ml for PRL. Intra- and interassay coefficients of variation for LH were 7.2% and 11.4%, respectively; for FSH were 8.0% and 13.2%, respectively; and for PRL were 8.1% and 11.4%, respectively.

Serum estradiol and progesterone were determined by RIA using specific antisera kindly provided by Dr. G. D. Niswender (Colorado State University, Fort Collins, CO) after ethyl ether extraction. Labeled hormones were purchased from Perkin-Elmer (Wellesley, MA). Assay sensitivities were 11.3 pg for estradiol and 500 pg for progesterone. Intra- and interassay coefficients of variation were 6.8 and 11.7 % for estradiol, respectively, and 7.1 and 12.15% for progesterone, respectively.

Statistics. Data are presented as means $\pm$ SE. Differences between treatment groups were estimated by one-way ANOVA followed by Tukey's post test using the Statistica Software. Frequency distributions were analyzed using the χ^2 test. $P < 0.05$ indicated statistically significant differences.

RESULTS

OX₁, OX₂, and PPO mRNA expression in ovaries of cycling females, and serum hormones and food intake. Both OX_1 and OX_2 mRNA expressions were detected in the ovary by real-
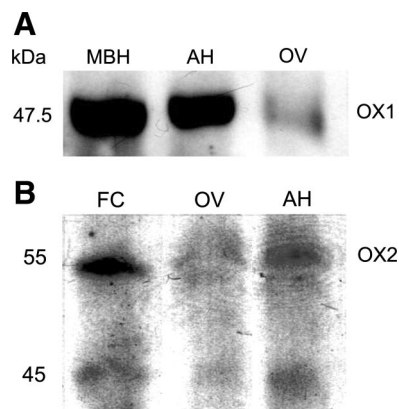

Fig. 2. *A*: Western blot analysis of OX_1 and OX_2 expression in membrane extracts of rat ovaries (OV). The antibody against OX_1 recognizes a band with an apparent molecular mass of 47.5 kDa, consistent with the expression of the native OX_1 receptor [according to NP_001516.1 and other authors (24, 29)] and another band of ∼35 kDa (not shown) also reported by Everest Biotech, UK, with a different anti-OX_1 antibody. MBH, mediobasal hypothalamus; AH, anterior hypothalamus. *B*: the anti-OX_2 receptor antibody also detected a specific band in ovary with and apparent molecular mass of 53 kDa [equivalent to the native OX_2 receptor, consistent with previous data (56)] and another band of ∼45 kDa also previously reported (30, 48). These bands were also found in positive control tissues: AH and frontoparietal cortex (FC).
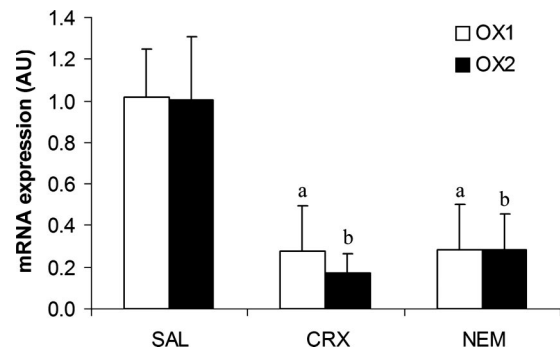

Fig. 3. Effect of Cetrorelix or Nembutal injected at 1400 on OX_1 and OX_2 mRNA expression examined at 1900 by real-time RT-PCR in ovaries of female proestrous rats. SAL, saline; CRX, Cetrorelix; NEM, Nembutal ($n = 8$ each). [a,b]Significantly different from SAL, $P < 0.05$. Both treatments blunted proestrous LH, FSH, PRL, and progesterone peaks (see text and Ref. 53).

Table 2. *Effects of orexin-1-selective (SB-334867-A) and orexin 2-selective (JNJ-10397049) receptor antagonists on ovulation and serum hormones in proestrous rats*

		Ova			Serum Hormones, ng/ml			
	n	%Ovulation	P	Ova Number	FSH Proestrus	FSH Estrus	LH	Prolactin
Vehicle	15	100	NS	15.21±0.50	7.23±0.62	4.06±0.29	12.75±0.85	93.57±10.15
SB-334867-A	8	75	<0.01	8.50±1.88†	3.84±0.40*	2.34±0.47*	9.14±1.38	77.81±7.92
JNJ-10397049	6	100	NS	9.00±1.26*	4.59±1.82	2.70±0.08*	4.63±1.59*	89.50±10.69
SB-334867-A + JNJ-10397049	9	56	<0.005	3.78±1.31‡	3.14±0.6*	2.97±0.50*	5.59±1.61*	82.92±4.58
CRX	10	0	<0.0001	0	2.96±0.23*	2.15±0.33*	0.36±0.07*	51.53±7.82*
NEM	15	0	<0.0001	0	2.40±0.29*	1.14±0.06*	0.34±0.06*	48.95±8.98*

Values are means ± SE. %Ovulation data were analyzed by the χ^2 test; ova number and serum hormone data were analyzed by ANOVA. Vehicle, ovulating controls; Cetrorelix (CRX) and Nembutal (NEM), nonovulating controls; FSH, follicle-stimulating hormone; LH, luteinizing hormone; NS, not significant. *$P < 0.05$, †$P < 0.002$, ‡$P < 0.001$.

time RT-PCR in diestrus 1 and 2, proestrus, and estrus. OX_1 mRNA expression peaked from 1700 to 2300 only during proestrus, and no changes were found at any other studied time (Fig. 1). A similar pattern was observed for OX_2 mRNA: highest expression in ovary between 1700 and 1900, while no changes were observed in any other stage of the cycle. No PPO mRNA expression was observed in the ovary on any day of the cycle at the studied times (not shown).

As previously described (53), LH, FSH, and PRL peaked during proestrous afternoon; estradiol and progesterone followed the expected patterns of our colony for this day of the cycle. As expected, food intake was always higher at night than during the day on each day of the estrous cycle (Table 1), marking a difference with OX_1 and OX_2 ovarian expression that only increased in the afternoon-night of proestrus.

To confirm the expression of orexin receptors at the protein level, Western blot experiments were performed in ovarian and control rat tissues (Fig. 2). Immunoblotting experiments using the anti-OX1R antibody (OX1R11-A) detected a specific band (47.5 kDa, equivalent to the native OX_1 receptor), in agreement with previous data (24), in the hypothalamus and ovary (Fig. 2A).

The anti-OX2R antibody (OX2R21A) detected a specific band in the hypothalamus, frontoparietal cortex, and ovary with an apparent molecular mass of 53 kDa [equivalent to the native OX_2 receptor, as previously reported (56)] and another band of ~45 kDa also previously reported (30, 48) (Fig. 2B).

Effect of blocking gonadotropin release by Cetrorelix or Nembutal on OX_1 and OX_2 expression in ovary. Cetrorelix or Nembutal administration early on proestrous afternoon significantly reduced the increase in ovarian OX_1 and OX_2 mRNA expression normally observed in the evening and night of proestrus (Fig. 3). Gonadotropins, PRL, and progesterone evening surges were also blunted by these treatments, while estradiol was unaffected (not shown), as demonstrated in our previous work in these models (53).

Effects of OX_1 and OX_2 receptor antagonists on gonadotropins, ovulation, and ovarian histology. All control animals ovulated the morning of estrus (Table 2), with a mean of 15.21 ± 0.50 ova/rat. Twenty five percent of rats treated with SB-334867-A, a selective OX_1 receptor antagonist, did not ovulate at all ($P < 0.01$), and in the remaining 75% a clear reduction in ova number was recorded (8.50 ± 1.88 ova/rat; $P < 0.01$ vs. vehicle-injected controls). SB-334867-A, injected at 1400 and 1900 of proestrus, reduced serum FSH at 1900 of proestrus and at 0900 of the following estrous morning (Table

2). Interestingly enough, in a preliminary study a single injection of this compound administered at 1400 also significantly reduced ova number at the same dose (10.0 ± 0.6 ova/rat, $n = 6$, $P < 0.02$). Food intake was reduced at both studied periods in these animals, indicating the effectiveness of SB-334867-A in this in vivo model (Table 3).

All rats injected at 1400 and 1900 with JNJ-10397049, a selective OX_2 receptor antagonist, ovulated, but a significant reduction in ova number was observed the following morning (Table 2). A significant reduction of LH during proestrous afternoon and of FSH on the following estrous morning was determined. Daytime food intake was also reduced (Table 3).

In the group of cycling female rats injected simultaneously with both orexin receptor blocking drugs, SB-334867-A plus JNJ-10397049, at 1400 and 1900 of proestrus, the blocking effect was more efficacious since four of nine rats did not ovulate at all (44%, $P < 0.005$), and the remaining five rats only ovulated 3.78 ± 1.31 ova/rat ($P < 0.001$). In this group, FSH and LH were reduced at all studied times (Table 2). A clear reduction in food intake was observed during the day (measured between 1400 and 1900) and at night (Table 3).

In Cetrorelix- and Nembutal-treated rats, used as controls of ovulation inhibition, ovulation was totally blocked, as expected, and serum FSH, LH, and PRL were significantly reduced (Table 2).

Anatomic examination of ovaries revealed profound differences among groups. In situ bloody ovaries were observed after laparotomy, mainly in rats injected with SB-334867-A (Fig. 4). This observation was confirmed by histology, where great abundance of red blood cells can be observed [Fig. 5, *top* (A–D) and *bottom* (E–H)]. Rats pretreated with SB-334867-A

Table 3. *Food consumption in rats pretreated with vehicle, CRX, NEM, and orexin-1-selective (SB-334867-A) and orexin-2-selective (JNJ-10397049) receptor antagonists*

	%Food Intake/Body Weight, g/g	
	1400–1900	1900–0900
Vehicle	0.845±0.082	4.141±0.168
SB-334867-A	0.114±0.059*	1.427±0.441*
JNJ-10397049	0.355±0.150*	3.441±0.400
SB-334867-A + JNJ-10397049	0.027±0.014†	1.618±0.250†
CRX	0.898±0.191	4.471±0.444
NEM	0.040±0.018†	2.169±0.284*

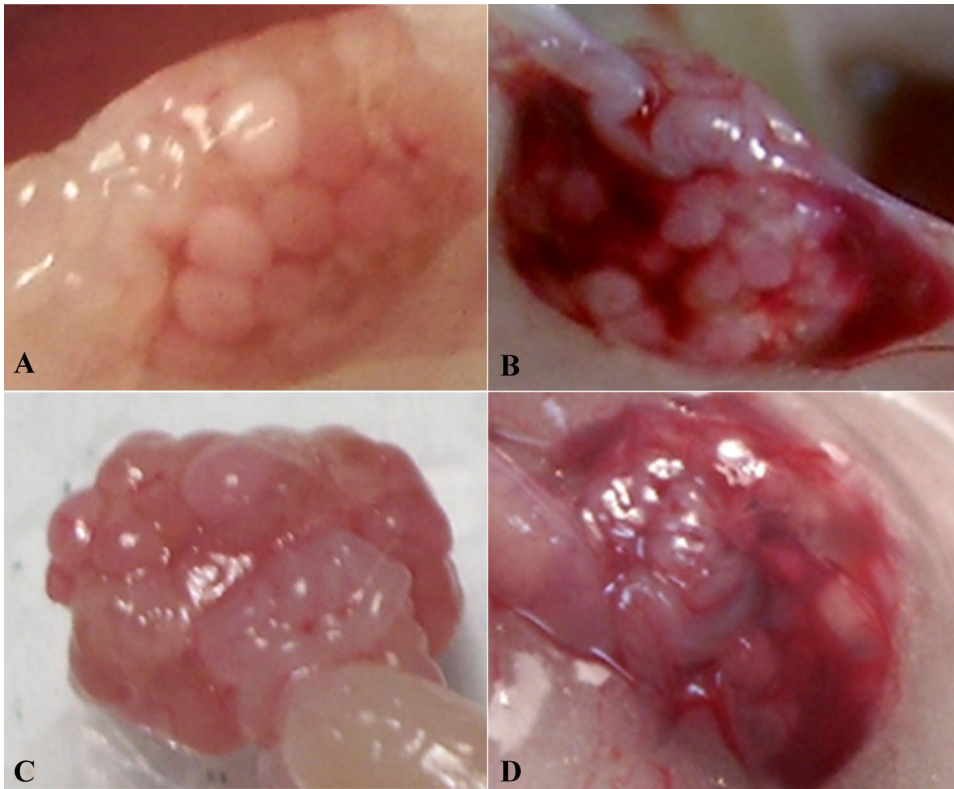Values are means ± SE. *$P < 0.01$, †$P < 0.001$.

Fig. 4. Photographs of in situ ovaries of vehicle- (*A*), SB-334867-A- (*B*), JNJ-10397049-(*C*), or SB-334867-A plus JNJ-10397049 (*D*)-injected rats. Note the bloody aspect of the ovaries, mainly in *B* and *D*.

and/or JNJ-10397049 showed more preovulatory follicles and less corpora lutea than controls (Fig. 6).

DISCUSSION

In a previous work, we described a particular relationship between the hormonal milieu of proestrus and the expression of components of the orexinergic system both at the hypothalamus and the pituitary (53). Here PPO, OX_1, and OX_2 expression was determined in ovaries of adult rats at different stages of the estrous cycle and correlated to the endocrine status, the dark-light cycle, and food consumption. Furthermore, the effects of two new potent and selective antagonists of orexin receptors were evaluated on gonadotropin secretion, ovarian histology, and ovulation. To our knowledge, this is the first description of the expression of both orexinergic receptors in this organ on each day of the estrous cycle, with a clear increase in proestrus. Previously, in a screening study, OX_1, but not OX_2, mRNA expression was observed in the gonad of rats using pooled ovaries (26).

Both orexinergic receptors vary along the estrous cycle, with a very marked increased observed in the evening of proestrus (OX_1 expression increases 3-fold, while OX_2 increases 4-fold). The fact that the increase of both orexin receptors' expression occurred exclusively during the late afternoon and night of proestrus strongly suggests that ovulation is related to this particular hormonal status and probably not related to the sleep-wake cycle or food intake.

To evaluate whether changes in the pituitary control of the gonad modified the expression of orexinergic receptors in the ovary, two of our models were used in proestrous animals (53). As expected, Cetrorelix and Nembutal blocked gonadotropin peaks; and, in addition, they reduced the expression of both

receptors OX_1 and OX_2 in the ovary. The fact that the increase in the expression of OX_1 and OX_2 was blunted in the ovary in proestrous animals injected with either agent suggests an important role for gonadotropins as regulators of this expression. In effect, in both models in which the action of the FSH and LH was impaired by different mechanisms, i.e., either GnRH was not properly released as in Nembutal-treated animals, or the effect of GnRH at the GnRH receptor was prevented by Cetrorelix, the proestrous ovarian OX_1 and OX_2 increases were completely abolished. Nevertheless, the participation of other hormones, such as, e.g., PRL or progesterone, in the regulation of ovarian orexin receptor expression cannot be disregarded, as their preovulatory increases were also abolished by Nembutal or Cetrorelix administration in our models (53).

Interestingly enough, no PPO mRNA expression was observed in the ovary, in agreement with data described by Jöhren et al. (26) in pooled ovaries, even when now analyzed throughout all the estrous cycle. Several hypotheses can be formulated to explain the presence of both receptors in the ovary in the absence of PPO; for example, the orexinergic peptide may originate elsewhere or the receptors may be activated by other molecules present in the gonad. The possibility that orexins may originate from outside the ovary and arrive by circulation is in line with the fact that immunoreactive orexin A has been described in human and rat plasma (2, 36). In this regard, PPO has been shown to be localized in discrete neurons in the lateral, perifornical, and dorsomedial hypothalamus (45). In addition, orexin immunoreactivity was reported in the rat median eminence and pituitary (13).

Furthermore, fluctuations of both orexins in hypothalamus during the estrous cycle were reported (49). Hypothalamic orexin A and B concentrations were reported to be higher in
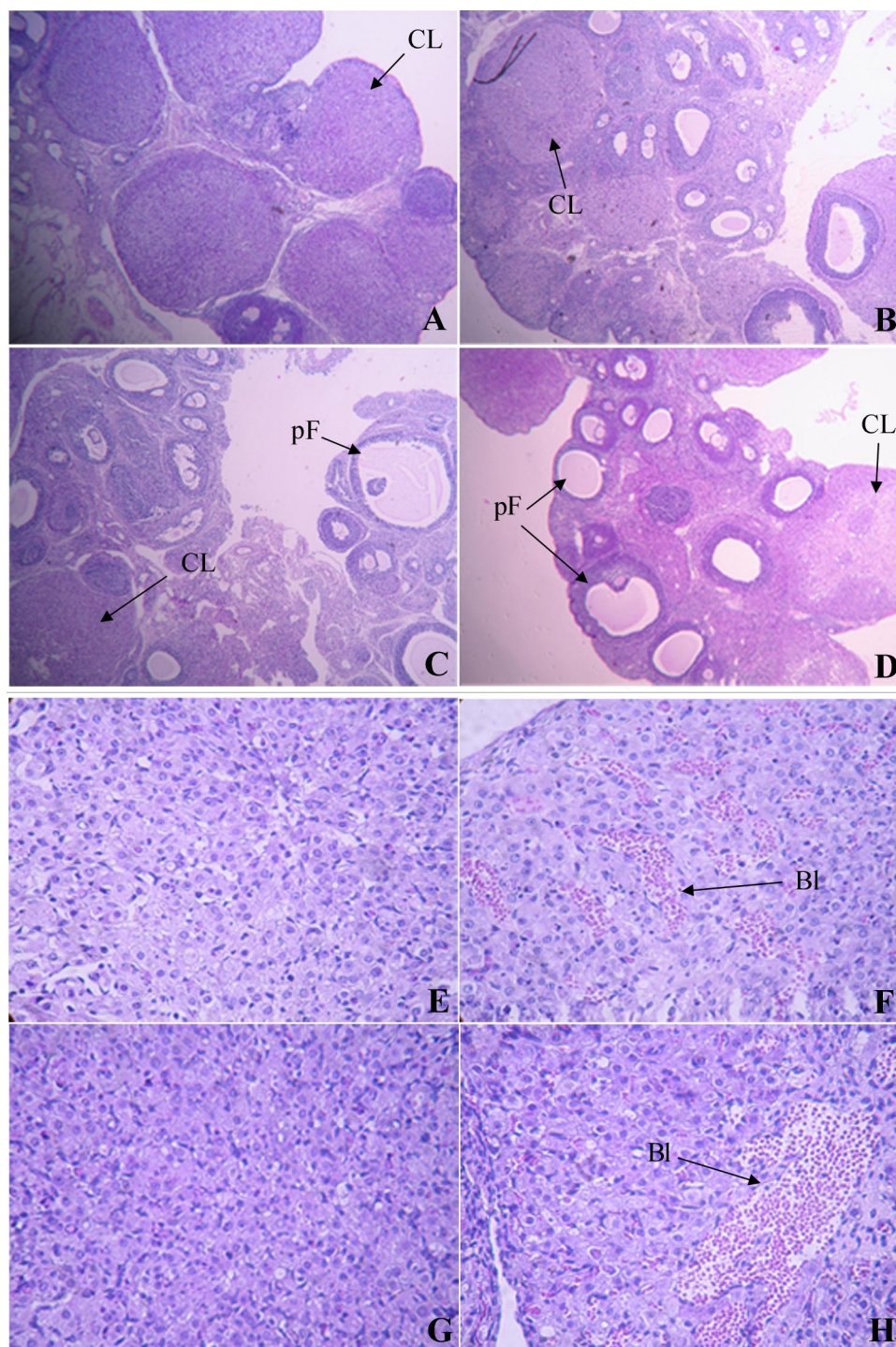
Fig. 5. *Top*: photomicrographs (×4) of ovaries in vehicle- (*A*), SB-334867-A- (*B*), JNJ-10397049- (*C*), or SB-334867-A plus JNJ-10397049 (*D*)-injected rats. *Bottom*: photomicrographs (×40) of corpora lutea in vehicle- (*E*), SB-334867-A- (*F*), JNJ-10397049- (*G*)- or SB-334867-A plus JNJ-10397049 (*H*)-injected rats. CL, corpus luteum; pF, preovulatory follicle; Bl, red blood cells.

proestrus than in diestrus in young cycling animals (46), and the greatest orexin A release from hypothalamus was suggested to occur on proestrus (46, 49). The possibility that orexins via general circulation reach the ovary and, alone or in combination with gonadotropins or other factors, may regulate OX_1 and OX_2 expression is an interesting hypothesis that should be the matter of further research.

Our next objective was to determine whether orexinergic blocking drugs modified serum gonadotropins, the expression of both receptors in the gonad and, as an end point, the occurrence of ovulation. Blocking OX_1 and/or OX_2 signaling inhibited gonadotropin secretion with particular effects for either agent. A single dose of SB-334867-A administered at 1400 reduced FSH release, without affecting LH, both measured at 1900 of proestrus; in contrast, a single dose of JNJ-10397049 at 1400 significantly diminished LH levels without modifying FSH. Evidently a single dose of one compound was not enough to drastically reduce both hormones. However, the combined effects of both agents decreased FSH and LH at 1900, suggesting that both receptors are involved in
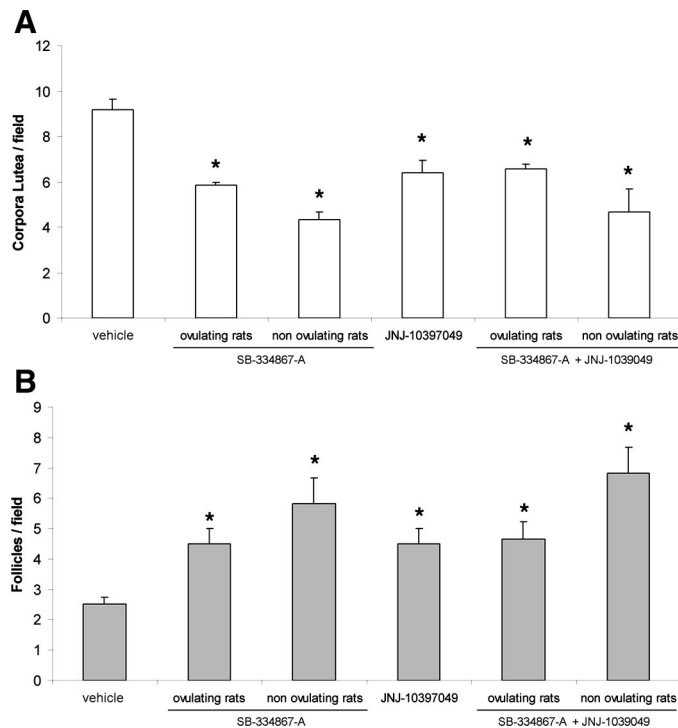
Fig. 6. Corpora lutea (*A*) and preovulatory follicles (*B*) per field in sections of ovaries from ovulating and nonovulating rats injected with vehicle, SB-334867-A, JNJ-10397049, or SB-334867-A plus JNJ-10397049. *Different from vehicle ($P < 0.05$).

the control of gonadotropin release and pointing to a participation of the hypothalamic orexinergic system in facilitating proestrous gonadotropin surges.

The lack of effect of both orexinergic antagonists on plasma PRL was an interesting finding that contrasts with the well-known blocking effect of Cetrorelix and Nembutal on the preovulatory peaks of the three hormones (53) and suggests that actions of SB-334867-A and JNJ-10397049 are more restricted to the GnRH-gonadotropin axis.

It is interesting to mention that a second injection of either blocking agent, SB-334867-A or JNJ-10397049, at 1900 of proestrus significantly reduced serum FSH in the morning of estrus. This second FSH peak has been reported to be independent of GnRH and due to a decrease in the secretion of ovarian inhibin, a well-known gonadal peptide able to inhibit FSH (11). In the present case, a drug-induced significant decrease in ovulation was observed (see below) that probably left preovulatory follicles secreting inhibin, reducing FSH levels in consequence.

These results using the new OX_1 and OX_2 blocking agents clarify previous results analyzing the effects of the orexinergic system on gonadotropin secretion. Some works explored the actions of orexins on GnRH and gonadotropin secretions in rodents. GnRH neurons express OX_1 receptor immunoreactivity and receive contacts from orexinergic axons (8). In vivo, intracerebroventricular injection of orexin A or orexin B rapidly stimulated LH secretion in a dose- and time-related fashion in estradiol benzoate- and progesterone-pretreated ovariectomized rats but inhibited LH release in unprimed rats, suggesting a modulation by estrogen of the response of the gonadotropins to orexins (18, 23, 47). Furthermore, orexins

injected into the third ventricle suppressed LH pulsatile secretion in ovariectomized rats (57) and an anti-orexin A antiserum suppressed preovulatory LH and PRL surges in the rat (32). In vitro, orexin A stimulated the release of GnRH in hypothalamic explants harvested from male rats and from females at proestrus, with no effect at estrus or metestrus; furthermore, orexin A inhibited GnRH-stimulated LH release in dispersed pituitaries from proestrous females (49). Orexin A also induces GnRH gene expression and secretion from GT1–7 neurons (51). In addition, a stimulatory effect of orexin A on LH release may involve direct actions on GnRH neurons (54). In sheep hypothalamus, immunohistochemistry indicated that orexin-containing neurons provide direct input to GnRH neurons (22). In humans, pulsatile LH release is diminished in orexin-deficient narcoleptic men (33). Therefore, our results blocking the orexin receptors in the afternoon of proestrus, together with all the previous data, clearly demonstrate the participation of the orexinergic system in the control of gonadotropin proestrous release. On the other hand, the reverse effect, i.e., the effects of the hormonal milieu on the orexinergic system, has also been studied, and changes in the orexinergic system have been associated with the estrous cycle, pregnancy, and lactation (5, 7, 19, 27, 46, 60). Recently, we described the effect of the proestrous environment on the expression of PPO and orexin receptors and in rat hypothalamus and pituitary (53).

Blocking orexin receptors early on proestrous afternoon had dramatic effects in the ovary. A significant decrease in the expected late proestrus increase of ovarian OX_1 and OX_2 expression was observed. Ovulation was markedly affected by these treatments: SB-334867-A administration induced a 25% reduction while the combination of both drugs induced a 44% reduction. In addition, treatments with either drug significantly decreased the number of ova released by the ovary, with the combined treatment being the most effective one. Furthermore, rats pretreated with SB-334867-A or JNJ-10397049 showed more preovulatory follicles and less corpora lutea than controls, in agreement with the decrease in the number of ova in the oviduct, pointing to an impediment in the ovulation process. Whether these effects respond to blockage of local receptors (direct effect) or whether they are due to inhibition of pituitary gonadotropins and/or hypothalamic orexins release (indirect effects), or a combination of both mechanisms, remains to be established.

Macro- and microexamination of ovaries on the morning of estrus revealed bloody ovaries, mainly in rats injected with SB-334867-A or a combination of both antagonists. This observation was confirmed by histology where the presence of large amounts of red blood cells could be observed. The effects of the OX_1 antagonist on the ovary were striking, possibly involving an effect on blood vessels. The link between orexins and the control of circulation has been suggested mostly at central levels, interacting with the autonomic system (9, 52) and affecting some local circuits as in the skeletal muscle (63), kidney (58), and brown adipose tissue (61), but the mechanism of action of this antagonist on ovarian rat vasculature and whether these erythrocytes are inside or outside the vasculature should be the matter of further research.

This surprising effect of SB-334867-A and the fact that 25% of rats treated with this drug did not ovulate at all may be indicative of different activities of each compound. Orexin A is

a more selective ligand for OX_1 while OX_2 binds both orexins A and B. Both receptor genes are widely expressed within the rat brain, but with some differences in distribution, and differential roles for OX_1 and OX_2 receptors have been suggested (1, 10, 59). Differential distribution of orexin A and orexin B immunoreactivity in the rat CNS has been described (12). Also, orexin A and B may be differentially released or cleared, as demonstrated in hypothalami of lactating rats (7), and orexin A but not orexin B rapidly enters brain from blood (31). Another aspect to take into consideration regarding the differential effects of both orexin receptor antagonists would be the specificity of the compounds. The OX_2 antagonist JNJ-10397049 has in vitro $\sim$600-fold selectivity for OX_2 over OX_1 (37); to our knowledge this is the first in vivo study published using JNJ-10397049. Although data seem to suggest that the effect on ovarian vessels is not OX_2 dependent and is either the result of OX_1 modulation by SB-334867-A or a consequence of the specificity of SB-334867-A, new studies remain to be done to indicate whether the differences observed between these new compounds depend on the pharmacology of them or to a differential function for each orexin and for each receptor in the ovary.

The feeding data clearly indicate no relationship between food intake and ovarian orexinergic receptors expression. The fact that food consumption was significantly lower in estrus than diestrus 2 is in agreement with previous results in several species, indicating lower intake around the time of ovulation (6, 16). Interestingly, SB-334867-A significantly reduced feeding during the day and night, while JNJ-10397049 significantly reduced feeding during the day, and night feeding was not significantly different from controls, suggesting the lack of feeding rebound; the combination of both drugs appeared to be significantly more efficacious than either given alone.

In summary, here we demonstrate for the first time the presence of both OX_1 and OX_2 mRNA in the ovary, their increased expression during the proestrous afternoon, and the dependence of this expression on the gonadotropins' peaks. Furthermore, two new synthetic compounds, SB-334867-A and JNJ-10397049, that selectively block OX_1 or OX_2, respectively, were tested. Both decreased gonadotropin release and ovulation, with profound macro- and microanatomic changes in the gonad.

REFERENCES

1. **Ammoun S, Holmqvist T, Shariatmadari R, Oonk HB, Detheux M, Parmentier M, Akerman KE, Kukkonen JP.** Distinct recognition of OX1 and OX2 receptors by orexin peptides. *J Pharmacol Exp Ther* 305: 507–514, 2003.

2. **Arihara Z, Takahashi K, Murakami O, Totsune K, Sone M, Satoh F, Ito S, Mouri T.** Immunoreactive orexin-A in human plasma. *Peptides* 22: 139–142, 2001.

3. **Barreiro ML, Pineda R, Gaytan F, Archanco M, Burrell MA, Castellano JM, Hakovirta H, Nurmio M, Pinilla L, Aguilar E, Topparí J,** Dieguez C, Tena-Sempere M. Pattern of orexin expression and direct biological actions of orexin-A in rat testis. *Endocrinology* 146: 5164–5175, 2005.

4. **Blanco M, Gallego R, Garcia-Caballero T, Dieguez C, Beiras A.** Cellular localization of orexins in human anterior pituitary. *Histochem Cell Biol* 120: 259–264, 2003.

5. **Brogan RS, Grove KL, Smith MS.** Differential regulation of leptin receptor but not orexin in the hypothalamus of the lactating rat. *J Neuroendocrinol* 12: 1077–1086, 2000.

6. **Buffenstein R, Poppitt SD, McDevitt RM, Prentice AM.** Food intake and the menstrual cycle: a retrospective analysis, with implications for appetite research. *Physiol Behav* 58: 1067–1077, 1995.

7. **Cai XJ, Denis S, Vernon RG, Clapham JC, Wilson S, Arch JR, Williams G.** Food restriction selectively increases hypothalamic orexin-B levels in lactating rats. *Regul Pept* 97: 163–168, 2001.

8. **Campbell RE, Grove KL, Smith MS.** Gonadotropin-releasing hormone neurons coexpress orexin 1 receptor immunoreactivity and receive direct contacts by orexin fibers. *Endocrinology* 144: 1542–1548, 2003.

9. **Ciriello J, McMurray JC, Babic T, de Oliveira CV.** Collateral axonal projections from hypothalamic hypocretin neurons to cardiovascular sites in nucleus ambiguus and nucleus tractus solitarius. *Brain Res* 991: 133–141, 2003.

10. **Cluderay JE, Harrison DC, Hervieu GJ.** Protein distribution of the orexin-2 receptor in the rat central nervous system. *Regul Pept* 104: 131–144, 2002.

11. **Culler MD, Negro-Vilar A.** Endogenous inhibin suppresses only basal follicle-stimulating hormone secretion but suppresses all parameters of pulsatile luteinizing hormone secretion in the diestrus female rat. *Endocrinology* 124: 2944–2953, 1989.

12. **Cutler DJ, Morris R, Sheridhar V, Wattam TA, Holmes S, Patel S, Arch JR, Wilson S, Buckingham RE, Evans ML, Leslie RA, Williams G.** Differential distribution of orexin-A and orexin-B immunoreactivity in the rat brain and spinal cord. *Peptides* 20: 1455–1470, 1999.

13. **Date Y, Mondal MS, Matsukura S, Ueta Y, Yamashita H, Kaiya H, Kangawa K, Nakazato M.** Distribution of orexin/hypocretin in the rat median eminence and pituitary. *Brain Res Mol Brain Res* 76: 1–6, 2000.

14. **de Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, Fukuhara C, Battenberg EL, Gautvik VT, Bartlett FS, Frankel WN, van den Pol AN, Bloom FE, Gautvik KM, Sutcliffe JG.** The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc Natl Acad Sci USA* 95: 322–327, 1998.

15. **de Lecea L, Sutcliffe JG, Fabre V.** Hypocretins/orexins as integrators of physiological information: lessons from mutant animals. *Neuropeptides* 36: 85–95, 2002.

16. **Dye L, Blundell JE.** Menstrual cycle and appetite control: implications for weight regulation. *Hum Reprod* 12: 1142–1151, 1997.

17. **Ferguson AV, Samson WK.** The orexin/hypocretin system: a critical regulator of neuroendocrine and autonomic function. *Front Neuroendocrinol* 24: 141–150, 2003.

18. **Furuta M, Funabashi T, Kimura F.** Suppressive action of orexin A on pulsatile luteinizing hormone secretion is potentiated by a low dose of estrogen in ovariectomized rats. *Neuroendocrinology* 75: 151–157, 2002.

19. **Garcia MC, Lopez M, Gualillo O, Seoane LM, Dieguez C, Senaris RM.** Hypothalamic levels of NPY, MCH, and prepro-orexin mRNA during pregnancy and lactation in the rat: role of prolactin. *FASEB J* 17: 1392–1400, 2003.

20. **Geerling JC, Mettenleiter TC, Loewy AD.** Orexin neurons project to diverse sympathetic outflow systems. *Neuroscience* 122: 541–550, 2003.

21. **Hara J, Beuckmann CT, Nambu T, Willie JT, Chemelli RM, Sinton CM, Sugiyama F, Yagami K, Goto K, Yanagisawa M, Sakurai T.** Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. *Neuron* 30: 345–354, 2001.

22. **Iqbal J, Pompolo S, Sakurai T, Clarke IJ.** Evidence that orexin-containing neurones provide direct input to gonadotropin-releasing hormone neurones in the ovine hypothalamus. *J Neuroendocrinol* 13: 1033–1041, 2001.

23. **Irahara M, Tamura T, Matuzaki T, Saito S, Yasui T, Yamano S, Kamada M, Aono T.** Orexin-A suppresses the pulsatile secretion of luteinizing hormone via beta-endorphin. *Biochem Biophys Res Commun* 281: 232–236, 2001.

24. **Irving EA, Harrison DC, Babbs AJ, Mayes AC, Campbell CA, Hunter AJ, Upton N, Parsons AA.** Increased cortical expression of the orexin-1 receptor following permanent middle cerebral artery occlusion in the rat. *Neurosci Lett* 324: 53–56, 2002.

25. **Johren O, Bruggemann N, Dendorfer A, Dominiak P.** Gonadal steroids differentially regulate the messenger ribonucleic acid expression of pituitary orexin type 1 receptors and adrenal orexin type 2 receptors. *Endocrinology* 144: 1219–1225, 2003.

26. **Johren O, Neidert SJ, Kummer M, Dendorfer A, Dominiak P.** Preproorexin and orexin receptor mRNAs are differentially expressed in peripheral tissues of male and female rats. *Endocrinology* 142: 3324–3331, 2001.

27. **Kanenishi K, Ueno M, Momose S, Kuwabara H, Tanaka H, Sato C, Kobayashi T, Hino O, Sakamoto H, Hata T.** Prepro-orexin mRNA expression in the rat brain is increased during pregnancy. *Neurosci Lett* 368: 73–77, 2004.

28. **Karteris E, Chen J, Randeva HS.** Expression of human prepro-orexin and signaling characteristics of orexin receptors in the male reproductive system. *J Clin Endocrinol Metab* 89: 1957–1962, 2004.

29. **Karteris E, Machado RJ, Chen J, Zervou S, Hillhouse EW, Randeva HS.** Food deprivation differentially modulates orexin receptor expression and signaling in rat hypothalamus and adrenal cortex. *Am J Physiol Endocrinol Metab* 288: E1089–E1100, 2005.

30. **Karteris E, Randeva HS, Grammatopoulos DK, Jaffe RB, Hillhouse EW.** Expression and coupling characteristics of the CRH and orexin type 2 receptors in human fetal adrenals. *J Clin Endocrinol Metab* 86: 4512–4519, 2001.

31. **Kastin AJ, Akerstrom V.** Orexin A but not orexin B rapidly enters brain from blood by simple diffusion. *J Pharmacol Exp Ther* 289: 219–223, 1999.

32. **Kohsaka A, Watanobe H, Kakizaki Y, Suda T, Schioth HB.** A significant participation of orexin-A, a potent orexigenic peptide, in the preovulatory luteinizing hormone and prolactin surges in the rat. *Brain Res* 898: 166–170, 2001.

33. **Kok SW, Roelfsema F, Overeem S, Lammers GJ, Frolich M, Meinders AE, Pijl H.** Pulsatile LH release is diminished, whereas FSH secretion is normal, in hypocretin-deficient narcoleptic men. *Am J Physiol Endocrinol Metab* 287: E630–E636, 2004.

34. **Kukkonen JP, Holmqvist T, Ammoun S, Akerman KE.** Functions of the orexinergic/hypocretinergic system. *Am J Physiol Cell Physiol* 283: C1567–C1591, 2002.

35. **Lang M, Soll RM, Durrenberger F, Dautzenberg FM, Beck-Sickinger AG.** Structure-activity studies of orexin A and orexin B at the human orexin 1 and orexin 2 receptors led to orexin 2 receptor selective and orexin 1 receptor preferring ligands. *J Med Chem* 47: 1153–1160, 2004.

36. **Lin J, Yan GT, Hao XH, Zhang K, Wang LH, Xue H.** Establishment and primary application of a highly-sensitive orexin-A radioimmunoassay. *J Immunoassay Immunochem* 25: 45–55, 2004.

37. **McAtee LC, Sutton SW, Rudolph DA, Li X, Aluisio LE, Phuong VK, Dvorak CA, Lovenberg TW, Carruthers NI, Jones TK.** Novel substituted 4-phenyl-[1,3]dioxanes: potent and selective orexin receptor 2 (OX(2)R) antagonists. *Bioorg Med Chem Lett* 14: 4225–4229, 2004.

38. **Mieda M, Yanagisawa M.** Sleep, feeding, and neuropeptides: roles of orexins and orexin receptors. *Curr Opin Neurobiol* 12: 339–345, 2002.

39. **Nakabayashi M, Suzuki T, Takahashi K, Totsune K, Muramatsu Y, Kaneko C, Date F, Takeyama J, Darnel AD, Moriya T, Sasano H.** Orexin-A expression in human peripheral tissues. *Mol Cell Endocrinol* 205: 43–50, 2003.

40. **Nambu T, Sakurai T, Mizukami K, Hosoya Y, Yanagisawa M, Goto K.** Distribution of orexin neurons in the adult rat brain. *Brain Res* 827: 243–260, 1999.

41. **Nowak KW, Strowski MZ, Switonska MM, Kaczmarek P, Singh V, Fabis M, Mackowiak P, Nowak M, Malendowicz LK.** Evidence that orexins A and B stimulate insulin secretion from rat pancreatic islets via both receptor subtypes. *Int J Mol Med* 15: 969–972, 2005.

42. **Olpe HR, Karlsson G, Pozza MF, Brugger F, Steinmann M, van Riezen H, Fagg G, Hall RG, Froestl W, Bittiger H.** CGP 53348: a centrally active blocker of GABA_B receptors. *Eur J Pharmacol* 187: 27–38, 1990.

43. **Parborell F, Irusta G, Vitale A, Gonzalez O, Pecci A, Tesone M.** Gonadotropin-releasing hormone antagonist antide inhibits apoptosis of preovulatory follicle cells in rat ovary. *Biol Reprod* 72: 659–666, 2005.

44. **Peluffo MC, Bussmann L, Stouffer RL, Tesone M.** Expression of caspase-2, -3, -8 and -9 proteins and enzyme activity in the corpus luteum of the rat at different stages during the natural estrous cycle. *Reproduction* 132: 465–475, 2006.

45. **Peyron C, Tighe DK, van den Pol AN, de Lecea L, Heller HC, Sutcliffe JG, Kilduff TS.** Neurons containing hypocretin (orexin) project to multiple neuronal systems. *J Neurosci* 18: 9996–10015, 1998.

46. **Porkka-Heiskanen T, Kalinchuk A, Alanko L, Huhtaniemi I, Stenberg D.** Orexin A and B levels in the hypothalamus of female rats: the effects of the estrous cycle and age. *Eur J Endocrinol* 150: 737–742, 2004.

47. **Pu S, Jain MR, Kalra PS, Kalra SP.** Orexins, a novel family of hypothalamic neuropeptides, modulate pituitary luteinizing hormone secretion in an ovarian steroid-dependent manner. *Regul Pept* 78: 133–136, 1998.

48. **Randeva HS, Karteris E, Grammatopoulos D, Hillhouse EW.** Expression of orexin-A and functional orexin type 2 receptors in the human adult adrenals: implications for adrenal function and energy homeostasis. *J Clin Endocrinol Metab* 86: 4808–4813, 2001.

49. **Russell SH, Small CJ, Kennedy AR, Stanley SA, Seth A, Murphy KG, Taheri S, Ghatei MA, Bloom SR.** Orexin A interactions in the hypothalamo-pituitary gonadal axis. *Endocrinology* 142: 5294–5302, 2001.

50. **Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, Arch JR, Buckingham RE, Haynes AC, Carr SA, Annan RS, McNulty DE, Liu WS, Terrett JA, Elshourbagy NA, Bergsma DJ, Yanagisawa M.** Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 92: 573–585, 1998.

51. **Sasson R, Dearth RK, White RS, Chappell PE, Mellon PL.** Orexin A induces GnRH gene expression and secretion from GT1–7 hypothalamic GnRH neurons. *Neuroendocrinology* 84: 353–363, 2006.

52. **Shirasaka T, Nakazato M, Matsukura S, Takasaki M, Kannan H.** Sympathetic and cardiovascular actions of orexins in conscious rats. *Am J Physiol Regul Integr Comp Physiol* 277: R1780–R1785, 1999.

53. **Silveyra P, Catalano PN, Lux-Lantos V, Libertun C.** Impact of proestrous milieu on expression of orexin receptors and prepro-orexin in rat hypothalamus and hypophysis: actions of Cetrorelix and Nembutal. *Am J Physiol Endocrinol Metab* 292: E820–E828, 2007.

54. **Small CJ, Goubillon ML, Murray JF, Siddiqui A, Grimshaw SE, Young H, Sivanesan V, Kalamatianos T, Kennedy AR, Coen CW, Bloom SR, Wilson CA.** Central orexin A has site-specific effects on luteinizing hormone release in female rats. *Endocrinology* 144: 3225–3236, 2003.

55. **Smart D, Sabido-David C, Brough SJ, Jewitt F, Johns A, Porter RA, Jerman JC.** SB-334867-A: the first selective orexin-1 receptor antagonist. *Br J Pharmacol* 132: 1179–1182, 2001.

56. **Takano S, Kanai S, Hosoya H, Ohta M, Uematsu H, Miyasaka K.** Orexin-A does not stimulate food intake in old rats. *Am J Physiol Gastrointest Liver Physiol* 287: G1182–G1187, 2004.

57. **Tamura T, Irahara M, Tezuka M, Kiyokawa M, Aono T.** Orexins, orexigenic hypothalamic neuropeptides, suppress the pulsatile secretion of luteinizing hormone in ovariectomized female rats. *Biochem Biophys Res Commun* 264: 759–762, 1999.

58. **Tanida M, Niijima A, Shen J, Yamada S, Sawai H, Fukuda Y, Nagai K.** Dose-different effects of orexin-A on the renal sympathetic nerve and blood pressure in urethane-anesthetized rats. *Exp Biol Med (Maywood)* 231: 1616–1625, 2006.

59. **Trivedi P, Yu H, MacNeil DJ, Van der Ploeg LH, Guan XM.** Distribution of orexin receptor mRNA in the rat brain. *FEBS Lett* 438: 71–75, 1998.

60. **Wang JB, Murata T, Narita K, Honda K, Higuchi T.** Variation in the expression of orexin and orexin receptors in the rat hypothalamus during the estrous cycle, pregnancy, parturition, and lactation. *Endocrine* 22: 127–134, 2003.

61. **Yasuda T, Masaki T, Kakuma T, Hara M, Nawata T, Katsuragi I, Yoshimatsu H.** Dual regulatory effects of orexins on sympathetic nerve activity innervating brown adipose tissue in rats. *Endocrinology* 146: 2744–2748, 2005.

62. **Yoshida K, McCormack S, Espana RA, Crocker A, Scammell TE.** Afferents to the orexin neurons of the rat brain. *J Comp Neurol* 494: 845–861, 2006.

63. **Zhang W, Sakurai T, Fukuda Y, Kuwaki T.** Orexin neuron-mediated skeletal muscle vasodilation and shift of baroreflex during defense response in mice. *Am J Physiol Regul Integr Comp Physiol* 290: R1654–R1663, 2006.